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Mutagenicity Test of Piroctone Olamine

(Experimental period: August 22 - 28, 1981)

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[April 27, 1982]

1-Hydroxy-4-methyl-6-(2,4,4-trimethylpentyl)-2(1H)-pyridone monoethanolamine salt (piroctone olamine; PO) synthesized by Hoechst Aktiengesellschaft, West Germany, is an antibacterial agent. This report deals with the results of the mutagenicity test (reverse mutation assay) of PO with Salmonella typhimurium and Escherichia coli strains.

Materials and Methods

Compound:

PO is a pale cream to white, odorless, fine crystalline powder and has a molecular weight of 298.42. Because of its insolubility in water, it was dissolved in dimethyl sulfoxide (DMSO, Merck, spectrophotometric grade), when used, to give the concentrations of 1, 2, 5, 10, 20, 50, 100, and 200 µg/plate.

Bacterial strains:

The strains used were S. typhimurium TA100, TA98, TA1535, TA1537, and TA1538 supplied kindly by Prof. Ames' laboratory, University of California, U.S.A., and E. coli WP2 uvrA⁻, by Prof. Ishizawa, Kyushu University, Japan. The TA series developed by Ames et al. [1,2] are the LT₂-derivative, histidine-requiring mutants which have high permeability for chemicals because of loss of the lipopolysaccharide barrier (rfa), and are sensitive to mutagens because of lack of UV excision repair (uvrB). TA1535 can be used to detect base-pair substitutions, and TA1537 and TA1538, to detect frameshift mutagens. TA100 and TA98 constructed by introducing an R factor plasmid, pKM101, into TA1535 and TA1538 are more sensitive to mutagenesis than the above 3 strains [2]. The E. coli strain used is a tryptophan-requiring mutant lacking UV excision repair (uvrA), which is usable for detection of base-pair substitutions.

For this experiment, each strain, which had been stored in a frozen state (-80°C), was inoculated into a nutrient broth culture on the day before use and was incubated with shaking at 37°C approximately for 16 hr to obtain bacterial suspension.

Preparation of S-9 and S-9 Mix:

The S-9 fraction was prepared with microsomes from the homogenized rat liver according to the method of Matsushima et al. [3]. Male Sprague-Dawley rats weighing approximately 200 g were treated as follows for enzyme induction. The animals received by the intraperitoneal (i.p.)

route 30 mg/kg of sodium phenobarbital (PB) (dissolved in physiological saline at a rate of 10 mg/ml) on the 1st day of experiment and 60 mg/kg each of PB (likewise, at a rate of 20 mg/ml) for the subsequent 3 days. On the 3rd day of this treatment period, they received 80 mg/kg, i.p., of 5,6-benzoflavone (dissolved in corn oil at a rate of 10 mg/ml) as well. On the next day of the last PB administration, the animals were sacrificed, and their livers were removed.

The liver was washed with 0.15 M KCl, placed in 3 volumes of 0.15 M KCl (3 ml/g of wet liver), and homogenized. The homogenate was centrifuged at 9000 xg for 10 min, and the supernatant served as the S-9 fraction. It was frozen in dry ice-acetone and quickly thawed when necessary. For this procedure used were all sterilized and cooled tools and solutions.

S-9 Mix was adjusted to consist of the following components per milliliter and used:

S-9 fraction	0.1 ml
MgCl ₂ (0.4 M) + KCl (1.65 M)	0.02
Glucose-6-phosphate (1 M)	0.005
NADPH (0.1 M)	0.04
NADH (0.1 M)	0.04
Sodium phosphate buffer (0.2 M)	0.5
H ₂ O	0.295

Minimal glucose agar plate:

Agar (60 g), glucose (80 g), and Vogel-Bonner minimal medium (400 ml) in separate bottles received 2800, 400 and 400 ml of distilled water, respectively. These 3 solutions were autoclaved, then mixed all together, and distributed into sterile Petri dishes (30 ml/dish) to make the agar plates.

The Vogel-Bonner minimal medium used contained the following components per liter of distilled water.

MgSO ₄ · 7H ₂ O	2 g
Citric acid	20
K ₂ HPO ₄	100
NH ₄ H ₂ PO ₄	19.2
NaOH	6.6

The medium prepared was autoclaved, stored in a frozen state, and thawed when used.

Top agar:

The top agar was made by mixing 10 volumes of an autoclaved aqueous solution of agar (0.6 wt%) and sodium chloride (0.5 wt%) and one volume of a sterile aqueous solution of L-histidine (0.5 mM)-biotin (0.5 mM) (0.5 mM tryptophan in place of L-histidine-biotin for the E. coli strain).

Test procedure:

The test was carried out by the preincubation method [4] as follows. Sterile test tubes received 50 μ l of the compound solution, 0.5 ml of sodium phosphate buffer or S-9 Mix, and 0.1 ml of each bacterial suspension in that order, and the tubes were preincubated with shaking at 30°C for 30 min. To each of them added was 2 ml of the top agar, and each mixture was spread evenly over the minimal glucose agar plate. After the agar hardened, the plates were incubated at 37°C for 48 hr, and the number of revertant colonies formed was counted.

The compound solution and the following positive controls were tested with 2 plates per concentration for each strain, and the solvent control, with 4 plates. The sterility tests of the solvent, S-9 Mix, and buffer were done with 2 plates.

Positive controls

2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2)
Benzo(a)pyrene (B(a)P)
N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)
2-Aminoanthracene (2AA)
9-Aminoacridine (9AA)
4-Nitroquinoline-1-oxide (4NQO)

Results

As Tables 1 - 6 show, bacterial growth was inhibited in any strain by the PO application at 100 and/or 200 μ g/plate in the presence and absence of S-9 Mix. In addition, in the case of TA100 and TA1535, growth was inhibited even at 50 μ g/plate. At 20 μ g/plate or less, the numbers of colonies formed were not different, regardless of the strain, from those in the solvent control plates.

Summary and Conclusion

PO was examined for its mutagenicity by the reverse mutation test with S. typhimurium TA100, TA98, TA1535, TA1537, TA1538 and E. coli WP2 uvrA⁻, which is known as the most available method for detection of mutagens of chemicals. The tests depended on the preincubation method, and were done with the PO concentrations of 1 - 200 µg/plate in both the cases with and without S-9 Mix.

At the highest 2 or 3 concentrations used, the PO application inhibited bacterial growth. At lower concentrations, however, the PO plates showed no abnormal increase in the number of revertant colonies of any strain formed, when compared with the solvent control plates.

From the results, PO is considered nonmutagenic in this test system.

References

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3. T. Matsushima, M. Sawamura, K. Hara, and T. Sugimura: In: In Vitro Metabolic Activation in Mutagenesis Testing (eds. F.J. de Serres, J.R. Fouts, J.R. Bend, and R.M. Philpot), pp. 85-88, North-Holland Biomedical Press, Amsterdam, 1976.
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Table 1 Effect of piroctone olamine on
S. typhimurium TA100

Compound concentration (μ g/plate)		No. of revertant colonies per plate	
		- S-9 Mix	+ S-9 Mix
Solvent control (DMSO)		(84, 69, 70, 95) ^{Mean} 80	(86, 86, 87, 94) ^{Mean} 88
1		(83, 91) 87	(82, 92) 87
2		(96, 96) 96	(87, 74) 81
5		(80, 70) 75	(84, 81) 83
10		(72, 62) 67	(93, 95) 94
20		(75, 80) 78	(84, 89) 87
50		(65, 66) 66	(56*, 62*) 59*
100		(35*, 44*) 40*	(28*, 33*) 31*
200		(Killing, Killing) Killing	(Killing, Killing) Killing
Positive control	Compound	AF-2	B (a) P
	Concentration (μ g/plate)	0.01	5
	No. of revertant colonies/plate	(508, 566) 537	(612, 684) 648

DMSO: Dimethyl sulfoxide

AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

B(a)P: Benzo(a)pyrene

* Bacterial growth inhibition was observed.

Table 2 Effect of piroctone olamine on
S. typhimurium TA98

Compound concentration (μ g/plate)		No. of revertant colonies per plate	
		- S-9 Mix	+ S-9 Mix
Solvent control (DMSO)		Mean (28, 28, 15, 33) 26	Mean (62, 67, 54, 42) 56
	1	(28, 28) 28	(48, 47) 48
	2	(28, 23) 26	(54, 46) 50
	5	(33, 24) 29	(58, 52) 55
	10	(24, 19) 22	(43, 56) 50
	20	(24, 21) 23	(71, 59) 65
	50	(30, 30) 30	(61, 43) 52
	100	(17*, 19*) 18*	(34, 52) 43
	200	(Killing, Killing) Killing	(Killing, Killing) Killing
Positive control	Compound	AF-2	B (a) P
	Concentration (μ g/plate)	0.1	5
	No. of revertant colonies/plate	(524, 568) 546	(312, 304) 308

DMSO: Dimethyl sulfoxide

AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

B(a)P: Benzo(a)pyrene

* Bacterial growth inhibition was observed.

Table 3 Effect of piroctone olamine on
S. typhimurium TA1535

Compound concentration (μ g/plate)		No. of revertant colonies per plate	
		- S-9 Mix	+ S-9 Mix
		Mean	Mean
Solvent control (DMSO)		(18, 13, 23, 18) 18	(15, 6, 7, 12) 10
	1	(12, 18) 15	(14, 9) 12
	2	(18, 18) 18	(10, 6) 8
	5	(14, 19) 17	(8, 12) 10
	10	(18, 15) 17	(9, 10) 10
	20	(16, 17) 17	(9, 15) 12
	50	(7*, 7*) 7*	(8, 7) 8
	100	(Killing, Killing) Killing	(4*, 4*) 4*
	200	(Killing, Killing) Killing	(Killing, Killing) Killing
Positive control	Compound	ENNG	2 AA
	Concentration (μ g/plate)	5	2
	No. of revertant colonies/plate	(1456, 1332) 1394	(139, 157) 148

DMSO: Dimethyl sulfoxide

ENNG: N-Ethyl-N'-nitro-N-nitrosoguanidine

2AA: 2-Aminoanthracene

* Bacterial growth inhibition was observed.

Table 4 Effect of piroctone olamine on
S. typhimurium TA1537

Compound concentration (μ g/plate)		No. of revertant colonies per plate	
		- S-9 Mix	+ S-9 Mix
		Mean	Mean
Solvent control (DMSO)		(5, 7, 8, 8) 7	(10, 10, 12, 15) 12
1		(4, 4) 4	(10, 14) 12
2		(7, 8) 8	(8, 11) 10
5		(3, 5) 4	(13, 16) 15
10		(4, 9) 7	(11, 18) 15
20		(6, 12) 9	(13, 14) 14
50		(5, 8) 7	(10, 13) 12
100		(5*, 1*) 3*	(10, 14) 12
200		(Killing, Killing) Killing	(Killing, Killing) Killing
Positive control	Compound	9 A A	B (a) P
	Concentration (μ g/plate)	80	5
	No. of revertant colonies/plate	(236, 258) 247	(118, 85) 102

DMSO: Dimethyl sulfoxide

9AA: 9-Aminoacridine

B(a)P: Benzo(a)pyrene

* Bacterial growth inhibition was observed.

Table 5 Effect of piroctone olamine on
S. typhimurium TA1538

Compound concentration (μ g/plate)		No. of revertant colonies per plate	
		- S-9 Mix	+ S-9 Mix
Solvent control (DMSO)		Mean (5. 6. 16. 16) 11	Mean (28. 27. 32. 36) 31
	1	(8. 15) 12	(31. 34) 33
	2	(10. 16) 13	(32. 35) 34
	5	(18. 16) 17	(28. 26) 27
	10	(11. 14) 13	(36. 28) 32
	20	(15. 17) 16	(35. 37) 36
	50	(10. 14) 12	(35. 31) 33
	100	(3*, 8*) 6*	(14*, 19*) 17*
	200	(Killing. Killing) Killing	(5*, 6*) 6*
Positive control	Compound	4NQO	B (a) P
	Concentration (μ g/plate)	0.25	5
	No. of revertant colonies/plate	(293. 314) 304	(144. 154) 149

DMSO: Dimethyl sulfoxide
4NQO: 4-Nitroquinoline-1-oxide
B(a)P: Benzo(a)pyrene

* Bacterial growth inhibition was observed.

Table 6 Effect of piroctone olamine on
E. coli WP2 *uvrA*⁻

Compound concentration (μ g/plate)		No. of revertant colonies per plate	
		- S-9 Mix	+ S-9 Mix
Solvent control (DMSO)		Mean (26, 21, 20, 24) 23	Mean (27, 23, 31, 26) 27
	1	(18, 20) 19	(30, 26) 28
	2	(17, 21) 19	(31, 25) 28
	5	(18, 21) 20	(21, 24) 23
	10	(16, 21) 19	(25, 28) 27
	20	(20, 22) 21	(33, 20) 27
	50	(18, 19) 19	(33, 20) 27
	100	(23, 15) 19	(30, 17) 24
	200	(Killing, Killing) Killing	(12, 14) 13 [*]
Positive control	Compound	AF-2	2 AA
	Concentration (μ g/plate)	0.01	5
	No. of revertant colonies/plate	(236, 212) 224	(796, 830) 813

DMSO: Dimethyl sulfoxide

AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

2AA: 2-Aminoanthracene

* Bacterial growth inhibition was observed.